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Stimulatory effects of lipoprotein(a) and low-density lipoprotein on human umbilical vein endothelial cell migration and proliferation are partially mediated by fibroblast growth factor-2

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Abstract

We previously reported a transient increase in plasma lipoprotein(a) (Lp(a)) concentrations following acute myocardial infarction and surgical operations, and demonstrated Lp(a) accumulation in healing tissues. In the present study, the stimulatory effect of Lp(a) on migration and proliferation of human umbilical vein endothelial cells (HUVEC) was assessed by quantitative assay methods and compared it with that of LDL. Lp(a) stimulated both migration and proliferation of HUVEC in a dose-dependent manner and the stimulatory activities for migration and proliferation were two times higher than those of LDL in terms of moles of apoB. In addition, this stimulatory activity of Lp(a) was not affected by the difference of Lp(a) phenotype. Although each neutralizing antibody to hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF) and interleukin-1β (IL-1β) had no further effect on migration and proliferation of HUVEC treated with Lp(a), only antibody to fibroblast growth factor-2 (FGF-2) partially suppressed them. Moreover, pertussis toxin, which inhibits FGF-2-stimulated endothelial cell movement, also partially suppressed Lp(a)-induced HUVEC migration. FGF-2 concentrations in the medium of HUVEC treated with Lp(a) were constant in spite of the increase in FGF-2 mRNA levels in HUVEC. Taken together, it is suggest that Lp(a) stimulates HUVEC migration and proliferation, which is mediated, at least in part, by FGF-2 and may promote the angiogenesis during wound healing.

Keywords: Lipoprotein(a); Low density lipoprotein; Human umbelical vein endothelial cell; Migration; Proliferation; Fibroblast growth factor-2

1. Introduction

Lipoprotein(a) (Lp(a)) is a macromolecular complex containing a unique glycoprotein, apolipoprotein (a) (apo(a)), linked by a disulfide bond to apoB-100 in low-density lipoprotein (LDL)-like particle, and increased plasma Lp(a) concentrations are associated with risk factors for atherosclerotic diseases. Although plasma Lp(a) concentrations are predominantly genetically determined with approximately 90% of the variation by the apo(a) gene, transient increases in plasma Lp(a) have been observed after acute attacks of myocardial infarction and surgical operations [1,2]. Recently, we have demonstrated immunohistochemically the existence of Lp(a) in the endothelial cells of small vessels and in the extracellular matrices around vessels in wounded tissues [3]. These findings suggest that Lp(a) may

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play a role in angiogenesis for healing of injured tissues.

Migration and proliferation of vascular endothelial cells (EC) are initiating processes in the repair of injured vessels and in the formation of capillaries. It is believed that migration is the rate-limiting process in the regeneration of endothelium, because migration is observed prior to proliferation [4,5]. These EC processes are involved in vascular reconstruction after balloon angioplasty [6–8]. Additionally, it is well known that EC produce a great variety of biologically active substances such as fibroblast growth factor-2 (FGF-2) [9,10], hepatocyte growth factor (HGF) [11], platelet-derived growth factor (PDGF) [12] and interleukin-1β (IL-1β). These growth factors act on cells in an autocrine and/or paracrine fashion, especially FGF-2 and HGF are potent stimulators for angiogenesis [10,11]. Takahashi et al. have recently demonstrated that the stimulatory ability of Lp(a) for proliferation of human umbilical vein endothelial cells (HUVEC) was 40% less than that of LDL in the presence of FGF-2 and insulin [13]. With regard to LDL, there are several reports showing that LDL is an agent for proliferation of EC [14,15]. Tauber et al. showed that LDL has a biphasic effect; mitogenic at low concentration and toxic at physiological concentration [14]. LDL has been shown to increase re-endothelialization in the wound by stimulating EC proliferation in vitro [15]. However, little is known about the effects of Lp(a) and LDL on EC migration.

In the present study, we investigated the effects of Lp(a) on both migration and proliferation of HUVEC in vitro by quantitative assay methods and compared them with those of LDL. In addition, the effects of several growth factors and cytokines on HUVEC treated with Lp(a) and LDL were also investigated to define the mechanisms for migration and proliferation.

2. Materials and methods

2.1. Cell culture

HUVEC were purchased from Kurabo (Osaka, Japan). Cells were cultured on collagen type I (50 μg/ml) (Upstate Biotechnology, Lake Placid, NY) coated plates in Medium 199 (M-199) (Nikken Biomedical, Kyoto, Japan) containing 10% fetal bovine serum (FBS) (Summit Biotechnology, Fort Collins, CO), 100 U/ml penicillin, 100 μg/ml streptomycin, 30 μg/ml endothelial cell growth supplement (Sigma, St. Louis, MO) and 20 μg/ml heparin (Nakarai Tesque, Kyoto, Japan) at 37°C in a 5% CO_2 environment.

2.2. Preparations of lipoproteins and apolipoproteins

Lp(a) and LDL were prepared from pool sera of normal blood donors. Sera were applied on dextran sulfate cellulose column (Kaneka, Osaka, Japan) and washed exhaustively with PBS (pH 7.4). The retaining fraction was eluted with 1 M NaCl containing 1 mM EDTA and 0.02% NaN_3 and dialyzed against PBS (pH 7.4). The density of 1.06–1.12 g/ml of this fraction was isolated by ultracentrifugation in a 50.2 Ti rotor (Beckman Instruments, Palo Alto, CA) and dialyzed against 20 mM Tris-HCl (pH 7.4). Dialyzed fraction was applied onto MonoQ HR 5/5 (Pharmacia, Uppsala, Sweden) strong anion exchanger column equilibrated with the same buffer [16,17]. Lp(a) was eluted from the column as a single peak with linear gradient of 1 M NaCl and was dialyzed against PBS containing 0.02% NaN_3 (pH 7.4). LDL was isolated in the density interval of 1.007–1.050 g/ml by sequential ultracentrifugation. The morphological uniformities of Lp(a) and LDL particles were confirmed by the negative stained electron microscopy [18]. The diameters of Lp(a) particles were from 266 to 297 Å with a mean diameter of 280 Å, which were slightly larger than LDL particles with the mean diameter of 244 Å (range 231–260 Å). Protein concentration of Lp(a) or LDL was determined by a DC Protein Assay kit (Bio-Rad, Hercules, CA), using bovine serum albumin as a standard and apoB concentration was measured by turbidimetric immunoassay (TIA) using a Hitachi-7150 automatic analyzer (Hitachi, Tokyo).

2.3. Immunological quantitation and phenotype classification of Lp(a)

Antibody to apo(a) was prepared as described previously [19]. This antibody did not show cross-reactivities against apoB-100, plasminogen and catalase

(Sigma, St. Louis, MO) [20]. Lp(a) concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using rabbit anti-apo(a) antiserum [19]. We used a commercial human Lp(a) (75 mg/l expressed in terms of total Lp(a), lot no. 290001, Immuno AG, Vienna, Austria) as the standard reference. Lp(a) phenotypes were categorized into seven phenotypes F, B, S1, S2, S3, S4 and null, in terms of relative mobility to apoB-100 according to a classification by Utermann et al. [21]. Our antibody used in this study is sensitive to all these Lp(a) phenotypes [22].

2.4. Migration assay

HUVEC migration was assayed using modified Boyden chamber reported by Grotendorst et al. [23]. This device is composed of 96-well microchemotaxis chambers with upper and lower wells (Neuro Probe, Cabin John, MD) and 5 μm-pore size polycarbonate filters (Nucleopore, Pleasanton, CA) between wells. After the filters were coated with collagen type 1 (50 μg/ml) for 30 min at 25°C, they were washed with PBS and then air-dried before use. Cultured HUVEC were trypsinized and suspended in M-199 containing penicillin and streptomycin. Eighty microliters of M-199 containing Lp(a) or LDL was applied in the lower wells and the cell suspension (2.0×10^4 cells per well) was placed on the upper wells. After a 4-h incubation at 37°C, the filter was removed and fixed with ethanol. Subsequently, cells were stained with Diff-Quick (Green-Cross, Tokyo, Japan). In this cell culture system, cells on the backside of the filter are considered to be migrated from the upper wells, though no cell actually transferred to the lower well during the period of cell culture. The numbers of migrated and unmigrated cells were counted in 3 fields per filter under a microscope (400× magnification). All assays were performed in quadruplicate and this method was highly reproducible. Migration activity was expressed as an average percentage of cell number on backside of the filter to total cell number on both sides.

2.5. Proliferation assay

The cultured HUVEC number was determined by a colorimetric assay that detects the change of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasolium bromide (MTT) (Chemicon, Temecula, CA) to violet-colored formazan crystals by mitochondria in cells as described by Kappel [24]. In brief, the trypsinized and washed HUVEC were seeded on 96-well plates coated with collagen type 1, at 5×10^3 cells per well. Medium was changed with FBS-free M-199 containing the test reagent after an overnight cell culture, and cells were incubated for 48 h at 37°C in a 5% CO_2 environment. After incubation, 10 μl of MTT was added to each well. Following further 4-h incubation at 37°C, 100 μl of 0.04 N HCl in isopropanol was added to dissolve the precipitated formazan. The absorbances were measured at a test wavelength of 570 nm and at a reference wavelength of 650 nm by an ELISA reader (Behring ELISA Processor II, Behringwerke, Marburg, Germany). Cell-free medium was used as blank. All assays were performed at least in triplicate. Proliferation activity was expressed as the ratio of sample absorbance to that of control (M-199 alone).

2.6. RNA extraction and measurement for FGF-2 mRNA

Total RNA was extracted with Isogen (Wako Pure Chemical Industries, Osaka, Japan). HUVEC were incubated with Lp(a) or LDL (50 μg protein/ml) in M-199 without FBS for 4 and 16 h. After incubation, harvested cells were suspended in Isogen (5×10^6 cells/ml), and 200 μl of chloroform was added. The samples were mixed vigorously and centrifuged at $12000\times g$ for 15 min. The extracted aqueous phase was added by 700 μl of isopropanol. After centrifugation, the pelleted RNA was washed with 75% ethanol, and then dissolved in TE. Total RNA amount was quantified with a spectrophotometer at 260 nm (GENE-QUANT, Pharmacia LKB Biochrom, Cambridge, UK).

Serial dilutions of 1, 2 and 4 μg of the total RNA denatured by 7% formaldehyde were applied onto a nylon membrane (Hybond N^+, Amersham, Arlington Heights, IL) for slot blot analysis. The quality of each RNA preparation was verified by Northern blot analysis. Thirty micrograms of total RNA of each sample was fractionated on 1.2% agarose gel containing 1.1% formaldehyde under denaturing conditions and transferred onto a nylon membrane. The

filters for slot blot and Northern blot analyses were baked at 80°C for 1.5 h under vacuum. The 40-mer oligonucleotides corresponded to human FGF-2 mRNA (Oncogene Science, Cambridge, MA) were labeled with [γ-^{32}P]ATP (Amersham) using an end-labeling kit (Megalabel, Takara, Kyoto, Japan). The hybridization and washing were performed at 45°C. Autoradiograms were developed following exposure to Hyperfilm-ECL (Amersham) at −70°C for an appropriate time and signals were scanned by a densitometer (Densito-pattern Analyzer EPA-300, Maruzen Chemical Oil Industries, Tokyo). The signal intensity was normalized with the density of ribosomal RNA.

FGF-2 concentrations in conditioned medium were determined by ELISA (R&D Systems, Minneapolis, MN).

2.7. Additional materials

Anti-human bFGF (FGF-2) IgG was purchased from Becton Dickinson Labware (Bedford, MA). Anti-human interleukin-1β (IL-1β) IgG was obtained from R&D Systems, anti-human PDGF-AB IgG from Upstate Biotechnology (Lake Placid, NY), anti-human HGF from Otsuka Pharmaceuticals (Tokyo, Japan) and recombinant FGF-2 from Boehringer Mannheim (Mannheim, Germany). Pertussis toxin was obtained from Gibco (Grand Island, NY).

2.8. Statistical analysis

Data are expressed as the mean ± S.D. Inter-group comparisons of mean values were performed by ANOVA, followed by post-hoc multiple comparison analysis with Fisher's least significant different test. Differences were considered significant if the P-value is <0.05.

3. Results

3.1. Effects of Lp(a) and LDL on migration and proliferation of HUVEC

HUVEC in serum-free medium were incubated with Lp(a) or LDL at indicated apoB concentrations for migration and proliferation studies. Migration

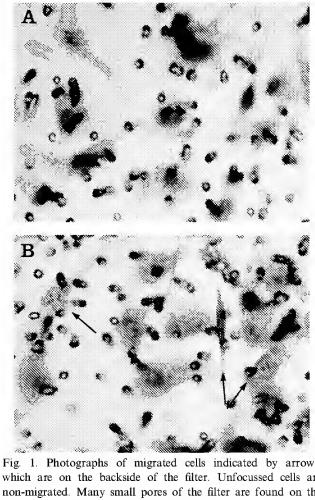

Fig. 1. Photographs of migrated cells indicated by arrows, which are on the backside of the filter. Unfocussed cells are non-migrated. Many small pores of the filter are found on the background. (A) control. (B) Lp(a). ×400.

activity of HUVEC was stimulated by Lp(a) and LDL, whereas it was significantly low (less than 1%) in control (Fig. 1 and Fig. 2A). Migration activities by Lp(a) and LDL were increased in a time-dependent manner for up to 12 h (data not shown). The migration activity by Lp(a) was increased in a dose-dependent manner and reached to approximately 10% at 10 μg/ml of apoB concentration. The stimulatory activity by Lp(a) was two times higher than that by LDL on the basis of apoB concentration.

HUVEC proliferation activities stimulated by Lp(a) and LDL were expressed as the ratio of activity to that of control. HUVEC treated with Lp(a) or LDL were proliferated in a dose-dependent manner, and the proliferation activities of Lp(a) and LDL were 3.3 and 3.4, respectively, at 10 μg/ml of apoB

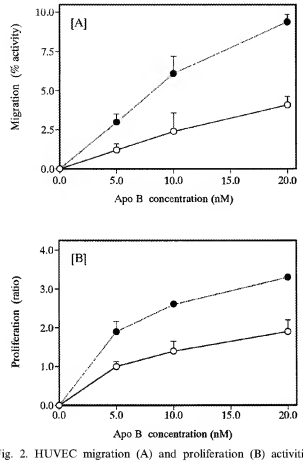

Fig. 2. HUVEC migration (A) and proliferation (B) activities treated with Lp(a) and LDL at indicated apoB concentrations. Lp(a) and LDL increased the migration and proliferation of HUVEC in dose-dependent manners. The effect of Lp(a) on HUVEC showed higher activity more than two times compared to LDL. ●, Lp(a); ○, LDL. Values are expressed as mean ± S.D. ($n = 4$).

concentration (Fig. 2B). The stimulatory activity by Lp(a) was approximately two times higher than that by LDL as well as in the migration.

3.2. Effect of Lp(a) phenotypes on migration and proliferation

Lp(a) phenotypes can be classified into at least seven types (F, B, S1, S2, S3, S4 and null) according to molecular size of apo(a). To investigate whether HUVEC migration and proliferation are affected by Lp(a) phenotype, we compared B type with S3/S4 type on apoB molar basis. The migration and proliferation of HUVEC were not significantly influenced by the difference of phenotype (Fig. 3).

3.3. Effects of neutralizing antibodies to various cytokines on HUVEC migration and proliferation treated with Lp(a) and LDL

To test whether HUVEC migration and proliferation are caused by the direct actions of Lp(a) and LDL, or are mediated via some other factors such as cytokines secreted from HUVEC themselves, we added neutralizing antibody to FGF-2, HGF, PDGF or IL-1β in the incubation medium containing Lp(a) or LDL. Migration activities of HUVEC stimulated by Lp(a) and LDL were suppressed to 52.4 and 48.3% of control, respectively, by the addition of 25 μg/ml of antibody to FGF-2 (Fig. 4A). Proliferations of HUVEC stimulated by Lp(a) and LDL were suppressed to 39.9 and 41.3% of control,

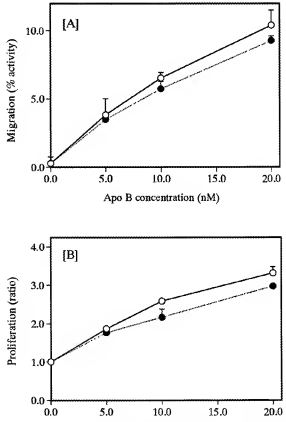

Fig. 3. The effects of Lp(a) phenotypes B and S3/S4 on HUVEC migration (A) and proliferation (B). No significant difference was observed in both experiments between two phenotypes. ●, phenotype S3/S4; ○, phenotype B. Values are expressed as mean ± S.D. ($n = 4$ for migration, $n = 3$ for proliferation).

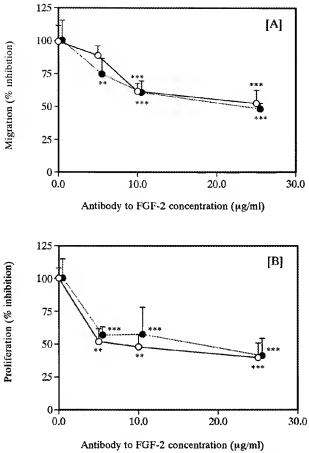

Fig. 4. The effects of anti FGF-2 antibody on HUVEC migration (A) and proliferation (B) stimulated by 50 μg protein/ml of Lp(a) and LDL. Antibody to FGF-2 suppressed the migration and proliferation stimulated by Lp(a) and LDL in a dose-dependent manner. ●, Lp(a); ○, LDL. Values are expressed as mean ± S.D. ($n=4$ for migration, $n=3$ for proliferation). $^{**}P<0.01$, $^{***}P<0.001$ compared to 0 μg/ml of antibody protein concentration.

respectively (Fig. 4B). The effect of antibody to FGF-2 alone was negligible for both migration and

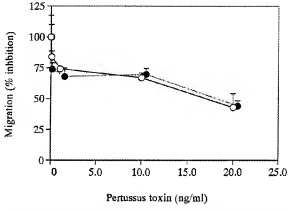

Fig. 5. The influence of pertussis toxin on HUVEC migration stimulated by Lp(a) and LDL. ●, Lp(a); ○, LDL. Values are expressed as mean ± S.D. ($n=4$).

proliferation. In contrast, antibody to HGF, PDGF or IL-1β had no further effect on HUVEC stimulated by Lp(a) or LDL (data not shown).

The effect of pertussis toxin, which is known to inhibit FGF-2-stimulated EC movement [25], on cell migration in the presence of 50 μg/ml Lp(a) or LDL was examined at the toxin concentrations of 0.1, 1.0, 10.0 and 20.0 ng/ml. As shown in Fig. 5, the cell migration was obviously suppressed dose-dependently. The addition of 20.0 ng/ml pertussis toxin decreased the migration activities stimulated by Lp(a) and LDL to 43 and 44% of control, respectively. The degrees of these inhibitions were compatible with those in our present experiment of neutralizing antibody to FGF-2 (see Fig. 4).

3.4. FGF-2 mRNA expression on HUVEC

FGF-2 mRNA levels in HUVEC treated with 50 μg/ml of Lp(a) or LDL for 4- and 16-h incubations are summarized in Table 1. Data are expressed

Table 1
The effects of Lp(a) and LDL on FGF-2 mRNA levels in HUVEC and FGF-2 levels in the medium

	FGF-2 mRNA (%)		FGF-2 concentration (pg/ml)	
	4 h	16 h	4 h	16 h
Control	100.0 ± 17.6	100.0 ± 12.8	1.6 ± 0.8	10.5 ± 1.3
Lp(a) (50 μg/ml)	157.8 ± 44.4*	171.8 ± 29.0**	1.8 ± 0.2	11.3 ± 2.0
LDL (50 μg/ml)	146.7 ± 17.6	141.2 ± 2.9*	1.7 ± 0.1	11.1 ± 2.9

Values are expressed as mean ± S.D. for three experiments.
$^{*}P<0.05$, $^{**}P<0.01$ compared with control at each time.

as the percentage of control at each incubation time. FGF-2 mRNA levels in HUVEC stimulated by Lp(a) and LDL significantly increased in both 4- and 16-h incubations compared to control, except for LDL in 4-h incubation. However, there were no significant differences between Lp(a) and LDL in both 4- and 16-h incubations. Northern blot analysis for FGF-2 mRNA showed high expressions of 4.0 and 1.9 kb and relatively lower expression of 7.0 kb of FGF-2 mRNA (data not shown), which were in agreement with a report of Malek et al. [26]. However, FGF-2 concentrations in the medium treated with Lp(a) or LDL were unaltered in either 4- or 16-h incubation.

4. Discussion

We and other investigators have reported that plasma Lp(a) level increases transiently after inflammation [1,2,27,28]. Recently, we demonstrated the localization of Lp(a) in the small vessels during healing process, indicating that Lp(a) may stimulate angiogenesis in wounded tissues [3]. It is known that EC migration and subsequent proliferation produce new blood vessels in wounded tissue [4–8]. To investigate the role of Lp(a) in angiogenesis, migration and proliferation were studied in HUVEC.

There are several lines of evidence that Lp(a) and LDL stimulate EC proliferation [13–15]. In this study, Lp(a) stimulated not only proliferation, but also migration in HUVEC in a dose- and time-dependent manner. Lp(a) showed a two-fold higher stimulatory activity than LDL on the basis of apoB concentration. This indicates that apo(a) has largely the same ability to stimulate as apoB, because the apo(a):apoB molar ratio is usually 1:1 in Lp(a). In contrast, Takahashi et al. reported that Lp(a) has less effect than LDL on HUVEC proliferation [13]. This discrepancy may be due to the different medium condition, because FGF-2 and insulin were contained in their medium. On the other hand, it has been reported that biological function of Lp(a) is not influenced by Lp(a) phenotype except for one report by Hervio et al. [29], showing that binding ability to fibrin varied with the phenotype. In the present study, no difference was observed in stimulatory activities for migration and proliferation, at least between phenotype B and S3/S4. This result is compatible with our previous observations that the magnitude of change in plasma Lp(a) concentrations during acute phase and the amount of Lp(a) deposition in wounded tissues are independent of Lp(a) phenotype [1,3].

In the current study, although each neutralizing antibody to HGF, PDGF and IL-1β had no further effect on the migration and proliferation of HUVEC stimulated by Lp(a) and LDL, only antibody to FGF-2 showed an inhibitory effect. This inhibitory effect of antibody to FGF-2 is partial, indicating that unknown mechanisms other than FGF-2 are also involved in HUVEC migration and proliferation induced by Lp(a) and LDL. Additionally, pertussis toxin which blocks EC movement mediated by FGF-2, suppressed Lp(a)- and LDL-induced HUVEC movement to the same extent as shown in our experiment of FGF-2 antibody. In these experiments (Figs. 4 and 5), Lp(a) and LDL largely showed the same response curves, although Lp(a) is twice as potent as LDL per molecule of apoB as shown in Fig. 2. This apparent contradiction is due to a different expression for lipoprotein quantity. Namely, Lp(a) and LDL were quantitated on the basis of apoB concentration in Fig. 2, but were on the basis of protein concentration in Figs. 4 and 5.

The present study provided the evidence that Lp(a) and LDL stimulate both migration and proliferation of HUVEC, which is mediated, at least in part, by FGF-2. However, FGF-2 concentrations in the medium were constant in spite of the increase in FGF-2 mRNA levels in HUVEC stimulated by Lp(a) and LDL. Although the reason for this apparent discrepancy remains unclear at present, the incubation time might be too short for the secretion of FGF-2. On the other hand, TGF-β, of which latent type is activated by plasmin, is known to be an inhibitor of smooth muscle cell and EC for growth and migration. Kojima et al. reported that Lp(a) inhibits the generation of activated TGF-β by suppressing plasminogen activation on cell surface [30]. Takahashi et al. have shown that TGF-β1 is not involved in the different potency to proliferate HUVEC between Lp(a) and LDL [13]. Although endogenous latent TGF-β can not be activated in our conditioned medium because of free of plasminogen, migration and proliferation were stimulated by Lp(a) or LDL.

Hence, it is unlikely that TGF-β involves in migration and proliferation of HUVEC in the present study. Furthermore, it is well known that native LDL is taken up by cells via LDL receptor [31]. In contrast to LDL, the catabolic pathway of Lp(a) is controversial, though there is a report showing that Lp(a) is weakly taken up via LDL receptor [32]. However, the precise mechanism has not been defined. Further studies are needed to elucidate other mechanisms than FGF-2-mediated pathway. In addition, it is known that the pathogenicity of Lp(a) for cardiovascular disease [33,34] may depend on the lysine-binding site (LBS) of Lp(a). However, the percentage of lysine-binding fraction of Lp(a) varies within the population [35,36]. Indeed, the percentage of lysine-binding fraction of Lp(a) is low in the Japanese (11.4 ± 5.7%) [37] and serum used in this study was less than 10%. Although it is important and interesting to ascertain whether these functions of Lp(a) for HUVEC depends on LBS, Lp(a) samples used here were unsuitable for such a kind of study.

In conclusion, Lp(a), whatever the phenotype, stimulates both migration and proliferation of HUVEC, which indicates the possibility that Lp(a) promotes angiogenesis during wound healing, and these stimulatory activities of Lp(a) for HUVEC were approximately two-fold higher than those of LDL in terms of moles of apoB.

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